NHE blockade inhibits chemokine production and NF-κB activation in immunostimulated endothelial cells

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Németh, Zoltán H., Edwin A. Deitch, Qi Lu, Csaba Szabó, and György Haskó. NHE blockade inhibits chemokine production and NF-κB activation in immunostimulated endothelial cells. Am J Physiol Cell Physiol 283: C396–C403, 2002. First published March 13, 2002; 10.1152/ajpcell. 00491.2001.—Na⁺/H⁺ exchanger (NHE) activation has been documented to contribute to endothelial cell injury caused by inflammatory states. However, the role of NHEs in regulation of the endothelial cell inflammatory response has not been investigated. The present study tested the hypothesis that NHEs contribute to endothelial cell inflammation induced by endotoxin or interleukin (IL)-1β. NHE inhibition using amiloride, 5-(N-ethyl-N-isopropyl)-amiloride, and 5-(N-methyl-N-isobutyl)amiloride as well as the non-amiloride NHE inhibitors cimetidine, clonidine, and harmaline suppressed endotoxin-induced IL-8 and monocyte chemoattractant protein (MCP)-1 production by human umbilical endothelial vein cells (HUVECs). The suppressive effect of amiloride on endotoxin-induced IL-8 production was associated with a decreased accumulation of IL-8 mRNA. NHE inhibitors suppressed both inhibitory (I)κB degradation and nuclear factor (NF)-κB DNA binding, suggesting that a decrease in activation of the IκB-NF-κB system contributed to the suppression of HUVEC inflammatory response by NHE blockade. NHE inhibition decreased also the IL-1β-induced HUVEC inflammatory response, because amiloride suppressed IL-1β-induced E-selectin expression on HUVECs. These results demonstrate that maximal activation of the HUVEC inflammatory response requires a functional NHE.

Na⁺/H⁺ exchanger; transcription factors; inflammation; cytokines; sepsis; adhesion molecule

NA⁺/H⁺ EXCHANGERS (NHEs) are a group of integral transmembrane proteins found in all mammalian cells and play a role in the regulation of intracellular pH, cell volume, vectorial ion transport, and cell proliferation (31). There is a large body of evidence demonstrating the presence of NHE activity in endothelial cells (2, 7, 12, 18, 19). NHEs in endothelial cells have been shown to be activated by a variety of stimuli, including acidosis (2, 19), hypoxia-reoxygenation (18), extracellular nucleosides (12), and nucleotides (7). Pharmacological inhibition of the increased activity of NHEs prevents endothelial cell injury and swelling induced

by acidosis (2, 19) and decreases the detrimental elevation of intracellular Ca²⁺ caused by hypoxia-reoxygenation (18), suggesting that NHE activation contributes to endothelial cell damage during acidosis and hypoxia-reoxygenation. In agreement with these in vitro observations, there is accumulating in vivo evidence that the activation of NHEs contributes to endothelial cell injury during a variety of pathophysiological processes associated with hypoxia and acidosis. For example, Mazzoni et al. (23) reported that the inhibition of NHEs by amiloride prevents capillary swelling and the consequent luminal narrowing during hemorrhagic shock. Furthermore, pharmacological blockade of NHEs prevents the increase in lung endothelial permeability induced by either septic shock (32) or hemorrhagic shock (24).

Pathophysiological states associated with hypoxia-reoxygenation and acidosis, such as shock and sepsis, are also characterized by an enhanced endothelial cell inflammatory response (20). During shock and sepsis, endothelial cells become chemoattractant, facilitating leukocyte adhesion, activation, and transmigration. There are numerous chemoattractant agents produced at the site of injury that participate in the recruitment of leukocytes, which include chemokines, platelet-activating factor, leukotriene B₄, the complement products C5a and C3a, and granulocyte-monocyte colony-stimulating factor. Chemokines are small cytokines, with molecular masses in the range of 8–12 kDa (39). Chemokine domains are defined by the presence of four cysteines in highly conserved position. One major chemokine subfamily is called "CXC" because the two amino acids nearest the NH₂ termini of these proteins are separated by a single amino acid. This is in contrast to the other major subfamily, which is called "CC" because these two cysteines are adjacent. The prototypic CXC chemokine is interleukin (IL)-8, which has been purified by several groups as a monocyte-derived factor that attracts neutrophils but not monocytes. IL-8 is also the major CXC chemokine secreted by endothelial cells. On the other hand, the CC chemokine monocyte chemoattractant protein (MCP)-1 is the most abundant chemokine released by endothelial cells. The

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most important inducers of chemokines in endothelial cells are bacterial products, such as bacterial lipopolysaccharide (LPS, endotoxin), as well as the monocyte/macrophage-derived cytokines tumor necrosis factor (TNF)-α and IL-1 (40).

Recently, NHEs have been implicated in the regulation of chemokine production. Inhibition of NHEs suppresses IL-8 production in respiratory epithelium infected with respiratory syncytial virus as well as in immunostimulated monocytes (21, 36). In addition, we have recently demonstrated that NHE inhibition suppresses macrophage inflammatory protein (MIP)-1α and MIP-2 production by LPS-stimulated macrophages (26). These observations suggest that NHE activation may be an important contributory factor to chemokine production during inflammatory cell activation. Thus we hypothesized that NHEs may be involved in the regulation of the endothelial cell chemokine response. The results of this study present evidence that, similar to other cell types, NHE activation is involved in the promotion of chemokine production in endothelial cells. Furthermore, our results demonstrate that one of the major intracellular targets of the proinflammatory effect of NHE activation is the inhibitory (I)κB-nuclear factor (NF)-κB system.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from BioWhittaker (Walkersville, MD) and cultured in endothelial growth medium. The HUVECs used for all experiments were from a Caucasian female umbilical cord. The cells were grown in a humidified atmosphere of 95% air-5% CO_2 at 37 °C. Second- to fourth-passage HUVECs were used in all experiments.

Drugs and reagents. The selective NHE inhibitors amiloride HCl, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), and 5-(N-methyl-N-isobutyl)amiloride (MIA) were obtained from Research Biochemicals (Natick, MA). The nonselective NHE inhibitors cimetidine, harmaline, and clonidine were purchased from Sigma (St. Louis, MO). All NHE inhibitors were dissolved in 0.5% DMSO. Human IL-1β was obtained from R&D Systems (Minneapolis, MN). LPS (*Escherichia coli* 055: B5) was purchased from Sigma. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Fisher Scientific (Pittsburgh, PA).

Chemokine measurement. To study the effect of NHE inhibitors, cells in 96-well plates were pretreated with these agents 30 min before stimulation with LPS (10 μg/ml). IL-8 and MCP-1 production were measured from supernatants taken 6, 12, 18, and 24 h after the LPS challenge. However, because both MIA and EIPA were toxic to the cells when the incubation lasted for 24 h, the effect of these agents on IL-8 and MCP-1 production was tested 4 h after LPS stimulation. Cimetidine, harmaline, and clonidine were administered to the cells 30 min before addition of LPS. The effect of these agents on IL-8 production was tested from supernatants obtained 8 h after LPS stimulation. Human IL-8 and MCP-1 levels in the supernatants were determined by using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions.

Western blot analysis for the assessment of IκBα degradation. HUVECs in six-well plates were pretreated with amiloride (300 μM) or vehicle (0.5% DMSO), and 30 min later the cells were stimulated with LPS (10 μg/ml) for 45 min. After

being washed with PBS, the cells were lysed by the addition of modified radioimmunoprecipitation buffer [50 mM Tris·HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4]. The lysates were transferred to Eppendorf tubes and centrifuged at 15,000 g, and the supernatant was recovered. Protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Sample (10 μg) was separated on a 8–16% Tris-glycine gel (Invitrogen, Carlsbad, CA) and transferred to a nitrocellulose membrane. The membranes were probed with anti-IκBα antibody (Cell Signaling, Beverly, MA) and subsequently incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Boehringer, Indianapolis, IN). Bands were detected by using enhanced chemiluminescence Western blotting detection reagent (ECL; Amersham Life Science, Arlington Heights, IL).

RNA isolation and RT-PCR. HUVECs in six-well plates were pretreated with amiloride (300 μM) or vehicle (0.5% DMSO), and 30 min later the cells were stimulated with LPS (10 μg/ml) for 4 h. Total RNA was isolated from HUVECs by using Trizol reagent (Invitrogen). RT of the RNA was performed by using MuLV reverse transcriptase (50 U/μl) from Perkin Elmer (Foster City, CA). RNA (5 μg) was transcribed in a 20-μl reaction containing 10.7 μl of RNA, 2 μl of 10× PCR buffer, 2 μl of 10 mM dNTP mix, 2 μl of 25 mM $MgCl_2$, 2 μl of 100 mM dithiothreitol (DTT), 0.5 μl of RNase inhibitor (20 U/μl; Perkin Elmer), 0.5 μl of 50 mM oligo d(T)$_{16}$, and 0.3 μl of reverse transcriptase. The reaction mix was incubated at 42°C for 15 min for reverse transcription. Thereafter, the reverse transcriptase was inactivated at 99°C for 5 min. RT-generated DNA was amplified by using the Expand high-fidelity PCR system (Boehringer Mannheim). The reaction buffer (25 μl) contained 1–5 μl of cDNA, water, 2.5 μl of 10× PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 0.5 μl of 10 mM oligonucleotide primer (each), and 0.2 μl of enzyme. cDNA was amplified by using the following primers and conditions: IL-8 (27), 5'-ATGACTTCCAAGC-TGGCCGTGGCT-3' (sense) and 5'-TCTCAGCCCTCTTCA-AAAACTTCTC-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CGGAGTCAACGGATTTGGT-CGTAT-3' (sense) and 5'-AGCCTTCTCCATGGTGGTGAA-GAC-3' (anti-sense). An initial denaturation occurred at 94°C for 5 min, with 27 and 23 cycles of 94°C for 30 s for IL-8 and GAPDH, respectively, 58°C for 45 s, 72°C for 45 s, and a final dwell at 72°C for 7 min. The expected PCR products were IL-8 (289 bp) and GAPDH (306 bp). PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

NF-κB electrophoretic mobility shift assay and supershift assay. After pretreatment with amiloride or its vehicle for 30 min, HUVECs in 75-ml tissue culture flasks were stimulated with LPS (10 μg/ml) for 45 min, and nuclear protein extracts were prepared as described previously (13, 25). All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with PBS and harvested by scraping into 1 ml of PBS and pelleted at 6,000 g for 5 min. The pellet was resuspended in one packed cell volume of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% vol/vol Nonidet P-40, 1 mM DTT, and 0.1 mM PMSF) and incubated for 5 min with occasional vortexing. After centrifugation at 6,000 g, one cell pellet volume of extraction buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% vol/vol glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing.

Nuclear proteins were isolated by centrifugation at 14,000 g for 15 min. Protein concentrations were determined with the Bio-Rad protein assay. Nuclear extracts were stored at $-70°C$ until used for electrophoretic mobility shift assay (EMSA). The oligonucleotide probe used for the EMSA was purchased from Promega. Oligonucleotide probes were labeled with $[\gamma\text{-}^{32}P]ATP$ by using T4 polynucleotide kinase (Invitrogen) and then purified in Bio Spin chromatography columns (Bio-Rad). For the EMSA analysis, 10 µg of nuclear protein were preincubated with EMSA buffer [12 mM HEPES, pH 7.9, 4 mM Tris·HCl, pH 7.9, 25 mM KCl, 5 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly(dI-dC), 12% glycerol vol/vol, and 0.2 mM PMSF] on ice for 10 min before addition of the radiolabeled oligonucleotide for an additional 25 min. The specificities of the binding reactions were tested by incubating duplicate samples with 100-fold molar excess of the unlabeled oligonucleotide probe. Protein-nucleic acid complexes were resolved by using a nondenaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:bisacrylamide) and run in 0.5× TBE (45 mM Tris·HCl, 45 mM boric aid, and 1 mM EDTA) for 1 h at constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under vacuum at 80°C for 1 h, and exposed to photographic film at $-70°C$ with an intensifying screen. For supershift studies, after addition of the radiolabeled probe, samples were incubated for 1 h with control, p65, or p50 antibody (Santa Cruz, CA) and then loaded on the gel.

Cell surface ELISA for the determination of E-selectin. HUVECs were seeded at 20,000 cells/well onto Biocoat Matrigel 96-well plates (Becton Dickinson, Bedford, MA) and used for the enzyme immunoassay protocols. To test the effect of NHE inhibition on HUVEC E-selectin expression, cells were pretreated with amiloride or its vehicle for 30 min. Subsequently, the cells were stimulated with 20 ng/ml IL-1β for 4 h. At the end of the incubation period, the supernatant was removed and the cells were washed twice with PBS and fixed with 1.5% paraformaldehyde for 30 min. Subsequently, the cells were blocked with 2% bovine serum albumin and then incubated for 1 h at 37°C with a monoclonal antibody to E-selectin (BD PharMingen, San Diego, CA). The cells were then incubated with an alkaline phosphatase-conjugated secondary antibody for 1 h. The cells were again washed three times, after which *p*-nitrophenyl disodium phosphate (1 mg/ml; Sigma) was added, and incubated at room temperature for 30 min. The colorimetric reaction was then read in a plate reader at 410 nm.

Measurement of mitochondrial respiration. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrion-dependent reduction of MTT to formazan. Cells in 96-well plates were incubated with MTT (0.5 mg/ml) for 60 min at 37°C. Culture medium was removed by aspiration, and the cells were solubilized in DMSO (100 µl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 550 nm using a Spectramax 250 microplate reader (17).

RESULTS

NHE inhibition suppresses IL-8 and MCP-1 production by HUVECs. First, we examined the effect of NHE inhibition on the production of IL-8 by HUVECs. Stimulation of HUVECs with LPS for 24 h increased IL-8 production by these cells from 1.26 ± 0.18 to 6.65 ± 0.26 ng/ml ($n = 6, P < 0.01$). Amiloride pretreatment of the HUVECs caused a dose-dependent, significant blunting of the IL-8 response to LPS measured at the

24-h time point (Fig. 1A). This inhibitory effect was not associated with any decrease in cell viability, as determined by the MTT assay (Fig. 1B). In the next set of experiments, we examined the effect of amiloride (300 µM) pretreatment on LPS-induced IL-8 production various time periods after LPS stimulation. Similar to results at the 24-h time point, amiloride also attenuated LPS-induced IL-8 production 12 and 18 h after stimulation with LPS (Fig. 1C). Importantly, the effect of amiloride was more pronounced at these early time points than at 24 h, because amiloride suppressed IL-8 production at 12 and 18 h by 61 and 54%, respectively, whereas the suppressive effect of amiloride amounted only to 30% at 24 h after the LPS challenge (Fig. 1C). Also similar to results at the 24-h point, amiloride failed to affect cell viability at any of the earlier time points tested (data not shown).

To further confirm the role of NHEs in the regulation of endothelial IL-8 production, we tested whether two
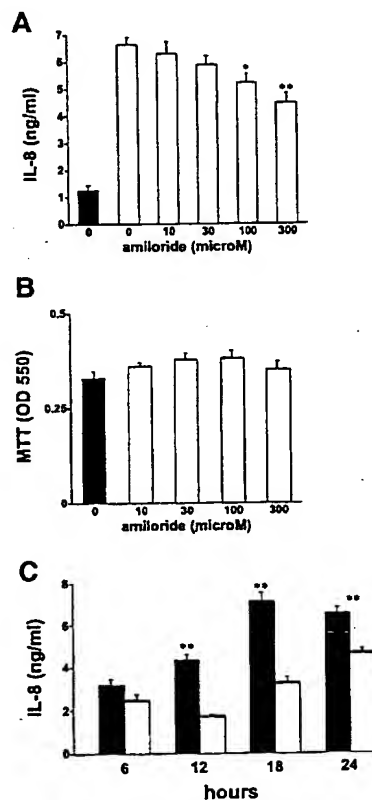

Fig. 1. *A*: amiloride suppresses lipopolysaccharide (LPS; 10 µg/ml)-induced interleukin (IL)-8 production by human umbilical vein endothelial cells (HUVECs). Filled bar, no LPS; open bars, LPS. Amiloride was added to the cells 30 min before stimulation with LPS. *B*: amiloride does not affect cell viability of HUVECs. Filled bar, no LPS; open bars, LPS. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *C*: amiloride suppresses IL-8 more effectively at 12 and 18 h after LPS than at 24 h after the LPS challenge. Filled bar, vehicle + LPS; open bars, amiloride + LPS. Supernatants for IL-8 measurement were taken 24 h after the LPS challenge. Data are means ± SE of $n = 12$ wells. *$P < 0.05$, **$P < 0.01$.

additional selective NHE inhibitors, EIPA or MIA, decreased the production of IL-8 after LPS stimulation. Figure 2A shows that both agents reduced the production of IL-8, confirming that NHE inhibition in endothelial cells has anti-inflammatory effects. Neither EIPA nor MIA was toxic to the cells as assessed with the MTT assay (Fig. 2B).

To further corroborate a role for NHEs in the regulation of IL-8 production, we next determined the effect of a series of NHE inhibitors that are structurally unrelated to amiloride on LPS-induced IL-8 production by HUVECs. These NHE inhibitors, which included cimetidine, harmaline, and clonidine (41, 47), all decreased LPS-induced IL-8 production by HUVECs (Fig. 3). The effect of all three non-amiloride NHE inhibitors was concentration dependent (Fig. 3). Under the conditions studied, none of these agents decreased cell viability at the concentrations tested (data not shown).

Finally, we assessed whether NHE inhibition by amiloride suppressed LPS-induced production of MCP-1. Similar to results for IL-8, LPS stimulated the release of MCP-1 into the medium (Fig. 4). Amiloride pretreatment of the cells decreased the LPS-stimulated MCP-1 level in the supernatant, which occurred in a dose-dependent manner (Fig. 4). The suppressive effect of amiloride on MCP-1 production was reproduced by using both EIPA and MIA (data not shown).
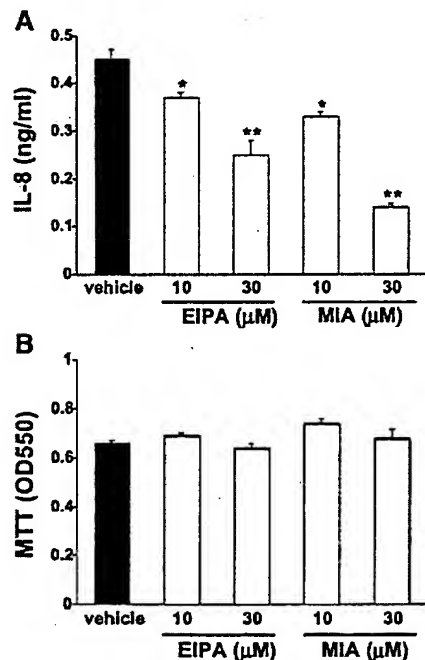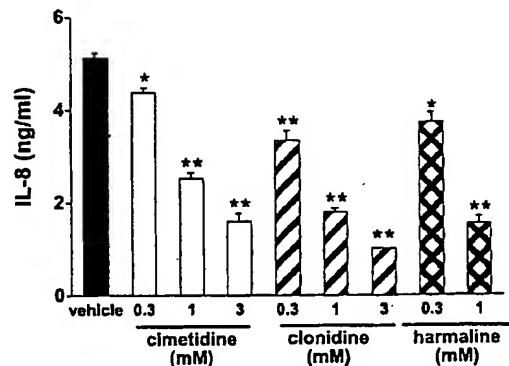

Fig. 3. Pretreatment of HUVECs with the non-amiloride NHE inhibitors cimetidine, clonidine, and harmaline reproduces the suppressive effect of amiloride analogs on the production of IL-8 by LPS (10 µg/ml)-induced HUVECs. Supernatants for IL-8 measurement were taken 8 h after the LPS challenge. Data are means ± SE of n = 12 wells. *P < 0.05, **P < 0.01.

These results confirm that NHE inhibition attenuates both IL-8 and MCP-1 production by HUVECs.

Amiloride suppresses LPS-induced IL-8 mRNA accumulation in HUVECs. To determine whether the suppressive effect of amiloride on IL-8 protein production was associated with an effect on the accumulation of IL-8 mRNA, we measured IL-8 mRNA levels from amiloride-pretreated HUVECs and controls. As shown in Fig. 5, LPS treatment for 4 h induced the accumulation of IL-8 mRNA. Amiloride pretreatment of the cells 30 min before the LPS challenge decreased the LPS-induced accumulation of IL-8 mRNA. GAPDH mRNA levels were not affected by amiloride treatment (Fig. 5), demonstrating that amiloride did not cause a general suppression of mRNA accumulation in HUVECs.

NHE inhibition inhibits LPS-induced NF-κB activation in HUVECs. Because the effect of amiloride was pretranslational, we hypothesized that amiloride may

Fig. 2. A: the amiloride analogs 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and 5-(N-methyl-N-isobutyl)amiloride (MIA) suppress LPS-induced IL-8 production by HUVECs. B: EIPA and MIA do not affect cell viability in HUVECs. MIA and EIPA were added to cells 30 min before stimulation with LPS (10 µg/ml). Supernatants for IL-8 measurement were taken 4 h after the LPS challenge. Data are means ± SE of n = 12 wells. *P < 0.05, **P < 0.01.
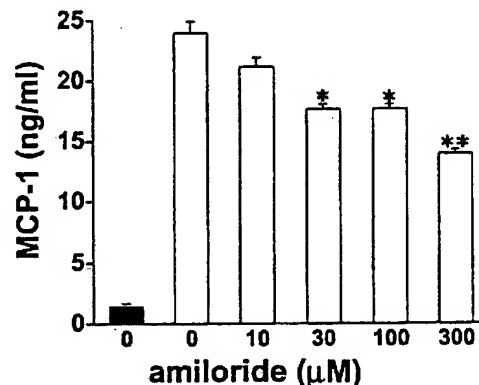

Fig. 4. Amiloride suppresses LPS (10 µg/ml)-induced monocyte chemoattractant protein (MCP)-1 production by HUVECs. Amiloride was added to the cells 30 min before stimulation with LPS. Supernatants for MCP-1 measurement were taken 24 h after the LPS challenge. Filled bar, no LPS; open bars, LPS. Data are means ± SE of n = 12 wells. *P < 0.05, **P < 0.01.
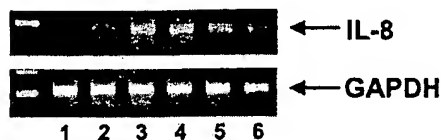
Fig. 5. Amiloride pretreatment (300 μM) 30 min before LPS (10 μg/ml) administration inhibits LPS-induced IL-8 mRNA accumulation in HUVECs. *Lanes 1* and *2*, vehicle; *lanes 3* and *4*, LPS; *lanes 5* and *6*, amiloride + LPS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were not affected by either LPS or amiloride treatment. IL-8 and GAPDH mRNA levels were quantified by using semiquantitative RT-PCR. Data are representative of 3 separate experiments.

Fig. 7. Amiloride pretreatment (300 μM) inhibits LPS-induced inhibitory (I)κB degradation in HUVECs. IκB degradation was assessed from cytosolic extracts obtained 45 min after LPS (10 μg/ml) stimulation. *Lanes 1* and *2*, vehicle; *lanes 3* and *6*, LPS; *lanes 4* and *5*, amiloride + LPS. Data are representative of 3 separate experiments.

have decreased the rate of transcription due to an effect on transcription factor activation. Because NF-κB is one of the most important transcription factors that mediates transcription of the IL-8 gene in response to LPS in HUVECs (6, 34, 35, 50), we first examined whether amiloride altered the binding of NF-κB to its consensus site by using the gel mobility shift assay. As shown in Fig. 6, using nuclear extracts from HUVECs treated with LPS, we observed a NF-κB DNA-binding complex that was not seen in LPS-nontreated cells (Fig. 6A). Supershift studies confirmed that the band induced by LPS contains both p65 and p50, because both the p50 and p65 antibodies shifted this complex (Fig. 6A). The increase in NF-κB binding caused by LPS was partially prevented when the cells were pretreated with amiloride 30 min before LPS treatment. This observation suggests that the mechanism of action of amiloride in suppressing IL-8 production involves an interference with the NF-κB pathway.

To further confirm a role for NHEs in the regulation of NF-κB activation, we next determined the effect of MIA and EIPA as well as cimetidine, clonidine, and harmaline on LPS-induced NF-κB DNA binding in HUVECs. Figure 6, *B* and *C*, demonstrates that, similar to amiloride, all of these NHE inhibitors decreased DNA binding of NF-κB.

It is well known that IκBα inhibits NF-κB translocation into the nucleus by sequestering NF-κB in the cytoplasm (35, 50). Thus we tested the possibility that amiloride suppressed NF-κB DNA binding by preventing IκB degradation and thereby decreasing NF-κB translocation into the nucleus. Western blot analysis was performed to examine steady-state IκBα protein levels in LPS-treated HUVECs in the presence or absence of amiloride. Figure 7 shows that LPS induced the degradation of IκBα, an effect that was prevented by pretreatment of the cells with amiloride. These results suggest that amiloride decreases NF-κB DNA binding by a mechanism that involves a suppressive effect on IκB degradation.

Amiloride suppresses IL-1β-induced E-selectin expression. We next determined whether blockade of NHEs by amiloride attenuated the expression of E-selectin. Stimulation of HUVECs with IL-1β caused a substantial upregulation of E-selectin expression (Fig. 8). Amiloride pretreatment of the cells 30 min before the IL-1β challenge attenuated the IL-1β-induced E-selectin response in a concentration-dependent manner (Fig. 8). This observation demonstrates that NHE inhibition decreases the IL-1β-induced HUVEC inflammatory response.

DISCUSSION

NHEs are a family of ubiquitous plasma membrane transport proteins that catalyze the exchange of extra-

Fig. 6. *A*: amiloride pretreatment (300 μM) inhibits LPS-induced nuclear factor (NF)-κB DNA binding in HUVECs. Ab, antibody. *B*: pretreatment with either MIA or EIPA reproduces the inhibitory effect of amiloride on LPS-induced NF-κB DNA binding. *Lane 1*, no LPS; *lane 2*, LPS; *lane 3*, EIPA + LPS; *lane 4*, MIA + LPS. *C*: cimetidine, clonidine, and harmaline also suppress NF-κB DNA binding. *Lane 1*, no LPS; *lane 2*, LPS; *lane 3*, cimetidine + LPS; *lane 4*, clonidine + LPS; *lane 5*, harmaline + LPS. NF-κB DNA binding was assessed from nuclear extracts obtained 45 min after LPS (10 μg/ml) stimulation. Data are representative of 3 separate experiments.
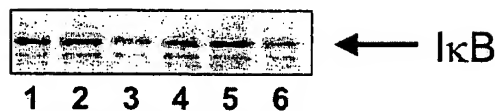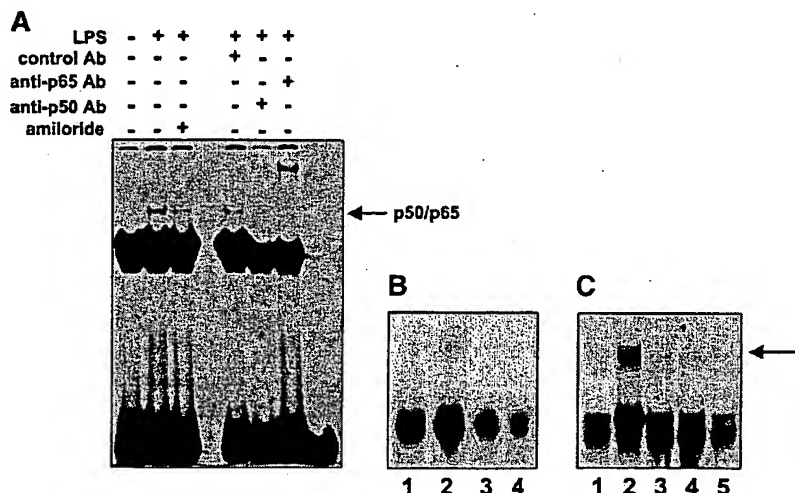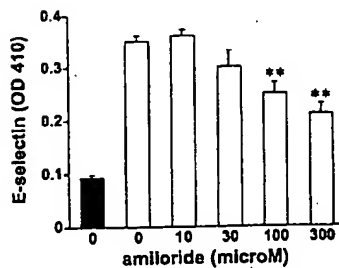
Fig. 8. Pretreatment of HUVECs with amiloride 30 min before stimulation with IL-1β (20 ng/ml) suppresses the upregulation of E-selectin. E-selectin expression 4 h after the LPS challenge was detected with ELISA. Filled bar, no IL-1β; open bars, IL-1β. Data are means ± SE. *$P < 0.05$, **$P < 0.01$.

cellular Na^+ for intracellular H^+ (31). However, NHEs have additionally been implicated in other cellular functions. For example, because they are stimulated by a variety of growth factors, a role in the initiation and/or control of mitogenesis has been proposed (16). In addition, NHEs are believed to be central to the maintenance and regulation of cell volume (49). In epithelial cells of the intestine and kidney, the apical NHE plays an important role in Na^+ reabsorption and acid secretion (9). Recent evidence indicates that NHEs regulate inflammatory processes. NHEs are rapidly activated in response to a variety of inflammatory signals, such as IL-1 (3, 5), TNF-α (43), interferon-γ (33), colony-stimulating factor 1 (42), and bacterial LPS (30, 43). This activation of NHEs by inflammatory stimuli crucially contributes to inflammatory responses of "professional" inflammatory cells, such as monocytes (30) and macrophages (33). These include TNF-α and IL-8 production (29, 30, 36), prostaglandin release (11), upregulation of Ia expression (33), Fcγ receptor expression (4), and colony-stimulating factor-induced proliferation (42). There is also evidence that the activation of NHEs is involved in promoting neutrophil migration (37) as well as myeloperoxidase activity and release (45). We recently demonstrated that inhibition of NHEs suppressed IL-12, MIP-1α, and MIP-2 production by LPS-stimulated macrophages (26). Furthermore, in the same study, we showed that inhibition of the Na^+/H^+ antiporter reduced plasma levels of IL-12 in an endotoxemic mouse model. Finally, inhibition of NHEs by amiloride suppresses IL-8 and IL-6 production in respiratory epithelium infected with respiratory syncytial virus (21).

Recent molecular cloning studies have confirmed that NHEs constitute a gene family from which seven mammalian isoforms (NHE1-7) have been cloned and sequenced (28, 31, 41). Characterization of the individual NHE isoforms has revealed differences in their primary structure, mode of regulation, cellular localization, and tissue expression. Despite the recent large expansion in our understanding of the molecular biology of NHEs, the most important tool to characterize the function of these proteins remains their pharmacological inhibition by the antidiuretic drug amiloride

and its analogs as well as several structurally unrelated molecules, including cimetidine, clonidine, and harmaline (41, 47). Although amiloride at low, nanomolar concentrations inhibits epithelial Na^+ channels (ENaCs) (22), effects that only appear at high amiloride concentrations in the micromolar range are thought to be exclusively selective for NHEs (31). The data presented here demonstrate, for the first time, that NHE activation contributes to the endothelial cell inflammatory response induced by LPS or IL-1β. The fact that amiloride and its analogs suppressed IL-8 and MCP-1 production in HUVECs with an IC_{50} value of 30–100 μM excludes a role for ENaCs in the amiloride suppression of endothelial cell inflammatory response. The idea that NHE inhibition suppresses the endothelial cell inflammatory response was confirmed by using three additional non-amiloride NHE inhibitors, clonidine, harmaline, and cimetidine, since all three of these inhibitors suppressed LPS-induced IL-8 production.

Cutaia et al. (8) have recently demonstrated that human pulmonary artery endothelial cells possess NHE1 but do not express NHE2–4. Furthermore, HUVECs have also been shown to express NHE1 (51); however, the expression of other subtypes has not been investigated. Nevertheless, it appears unlikely that NHE1 or NHE2 is the target of the suppressive effect of NHE inhibitors on inflammatory function in HUVECs. Both NHE1 and NHE2 are inhibited by amiloride concentrations not exceeding 1 μM (41, 47), and the IC_{50} values for NHE1 and NHE2 inhibition by EIPA and MIA are in the nanomolar range (41, 47). However, in our study, neither amiloride nor its analogs suppressed chemokine production at concentrations <10 μM. Thus it is improbable that either NHE1 or NHE2 is responsible for the attenuation of chemokine production by amiloride or its analogs. Interestingly, both amiloride and EIPA blocked thrombin-induced platelet-activating factor production by HUVECs at concentrations comparable to those that suppressed IL-8 production in our study (14). These results confirm the notion that HUVECs express amiloride-insensitive NHEs that regulate inflammatory processes. This view is further supported by the observation that harmaline, cimetidine, and clonidine decreased IL-8 production with IC_{50} values between 0.3 and 3 mM, which are higher than would have been expected if the NHE1 or NHE2 was involved (41, 47). Because NHE5 is also sensitive to nanomolar concentrations of EIPA (41, 47), it appears unlikely that this isoform plays a role in the regulation of IL-8 production. On the other hand, because both NHE3 and NHE4 have been documented to be inhibited by amiloride or non-amiloride compounds with a similar potency (41, 47) to that observed for the suppression of IL-8 production by HUVECs, NHE3 or NHE4 could be the subtype mediating the inhibitory effect of NHE inhibitors on IL-8 production. Because the NHE6 and NHE7 isoforms have not been characterized pharmacologically, their role in the regulation of endothelial cell IL-8 production remains to be elucidated.

Another important finding of the present study is that the suppressive effect of NHE inhibition on IL-8 production appears to have a pretranslational component, because amiloride suppressed LPS-induced IL-8 mRNA levels. This idea is supported by the observation that the effect of amiloride on IL-8 production was more pronounced early after LPS stimulation than at the 24-h time point. However, it is unlikely that the Golgi-related early secretion of IL-8 from the Weibel-Palade bodies (46) could be the target of NHE inhibition, because NHE inhibition failed to suppress IL-8 production 6 h after LPS stimulation.

We believe that the demonstration of the decreasing effect of NHE inhibition on the IκB-NF-κB system is a major step toward answering the question of how NHEs regulate endothelial cell or other inflammatory responses. The importance of NF-κB as a central mediator of the immunoregulatory effects of NHEs is highlighted by the fact that NF-κB activation is also under the control of NHEs in other cell types, such as the intestinal epithelial cell line HT-29 (25) and respiratory epithelial cells (15). These findings raise the important question of how a signal provided by the membrane protein NHE is transmitted to the cytosolic protein NF-κB. One of the most intriguing possibilities is that it is the alteration of cytoskeletal organization of actin filaments that links NHEs to NF-κB activation. This possibility is supported by the fact that NHEs are important regulators of actin filament assembly (10, 44, 48) and that changes in the actin microfilament system are involved in activation of the NF-κB system (1, 52). In this respect, it is important to note that NHE3 (38, 44, 48) has been reported to regulate cytoskeletal organization. The tethering of actin filaments to the plasma membrane NHE3 is mediated in part by the ezrin, radixin, and moesin (ERM) family of actin-binding proteins (38, 44, 48). Clearly, further studies are necessary to delineate the mechanisms whereby NHEs couple extracellular inflammatory signals to activation of the intracellular inflammatory cascade and NF-κB.

In summary, we have demonstrated that NHE activation contributes to the endothelial cell inflammatory response to inflammatory stimuli. We speculate that the mechanism of NHE promotion of inflammatory processes may have evolved as a positive feedback signal during endothelial cell activation.

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